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Treatment of viral infections

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Treatment of Viral Infections

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Treatment of Viral Infections

Description

The present invention relates to the use of inhibitors of the TRAIL ligand/TRAIL receptor system for the manufacture of a medicament for the prevention or treatment of viral diseases, particularly for the prevention or treatment of influenza or Borna disease virus infections.

NF-κB is activated by multiple families of viruses, including HIV-1, HTLV-1, Hepatitis B and C viruses, EBV, VSV and influenza viruses[1]. While for some of these viruses, e.g. retroviruses or oncogenic viruses, activation of this transcription factor may support viral replication, it is a common view that NF-κB acts in an antiviral fashion upon infection with RNA viruses, such as VSV or influenza virus[2,3]. RNA virus infections commonly result in activation of an innate antiviral response mediated by type I interferons (IFNs). This antiviral program is initiated by viral induction of the IFNß gene through constitutively expressed transcription factors, namely AP-1, IRF-3 and NF-κB[4]. Besides IFNß, also other genes involved in the induction of inflammation and immune responses are also regulated by NF-κB, such as IL-6, TNF-α or IL-12. Accordingly, VSV-induced expression of IFNß, IL-6 and IL-12 is impaired in cells deficient for the NF-κB activator IκB-kinase 2 (IKK2)[2].

Another level of NF-κB interference with virus propagation is through its capability to regulate apoptosis. NF-κB is mainly regarded as a survival factor by up-regulating antiapoptotic genes, such as Bcl-XL, A20 or cIAPs[5,6]. However, NF-κB was also reported to act proapoptotically under certain conditions, e.g. by upregulating the death-inducing CD95 ligand and its receptor[5,6]. Thus, the concept of a context-dependent regulation of apoptosis by NF-κB has emerged[6]. For example, Dengue virus-infected

HepG2 hepatocytes undergo apoptosis in an NF-κB- dependent manner[7] while NF-κB activation induced by the Hepatitis C virus core protein protects cells from apoptosis in the same cell line[8].

A common viral inducer of NF-κB-dependent responses appears to be double-strand RNA (dsRNA). Most RNA viruses produce dsRNA-like replication intermediates representing a shared molecular pattern that may be sensed by the cell as an alert signal[9].

Activation of IκB kinase (IKK) and NF-κB by influenza virus or influenza viral products is well documented[10-13], however, the function of this signalling module in the virus-infected cell is not as clear yet. The knowledge so far is restricted to data obtained with a recombinant virus termed delNS1 with a deletion of the complete coding region of the viral non-structural protein 1 (NS1) 14. Infection of cells with influenza delNS1 virus results in enhanced NF-κB activation and IFNß production[3] as well as enhanced apoptosis[15], suggesting that the NS1protein is, at least a partial antagonist of these presumed antiviral responses. Nevertheless, the consequences of influenza virus-induced NF-κB activation for the outcome of an infection has never been directly addressed yet.

According to the present application, it was found that NF-κB dependent viral induction is mediated via the proapoptotic factor TRAIL which enhances virus propagation in an auto- and paracrine fashion, particularly in the context of an influenza virus infection.

A first aspect of the present invention relates to the use of inhibitors of the TRAIL ligand/receptor system for the manufacture of a medicament for the treatment of viral infections, particularly for the treatment of viral infections caused by RNA viruses e.g. negative strand RNA viruses such as influenza viruses and Borna disease viruses. Especially preferred is the prevention or treatment of influenza virus infections. The TRAIL ligand/TRAIL receptor

inhibitors are used for the prevention or treatment of viral infection in humans or in domestic or wild animals, e.g. horses or sheep. The inhibitors of the present invention may be administered for the prevention of viral infections and/or for the treatment of acute or chronic viral infections.

In a preferred embodiment of the invention, the inhibitor is a TRAIL (TRAIL ligand APO-2) inhibitor. For example, TRAIL inhibitors may be selected from

(a) an inhibitory anti-TRAIL-antibody or a fragment thereof, and

(b) a soluble TRAIL-receptor molecule or a TRAIL-binding portion thereof.

Preferred are inhibitory anti-TRAIL-antibodies and antigen binding fragments thereof and soluble TRAIL receptor molecules or TRAIL ligand binding portions thereof. Examples of suitable anti-TRAIL antibodies are monoclonal antibodies, chimeric, humanized or human antibodies or antibody fragments, e.g. proteolytic fragments or recombinant single-chain fragments. These antibodies may be obtained by immunization and selection procedures known in the art. Further preferred are soluble TRAIL receptor molecules, e.g. a soluble TRAIL receptor molecule without the transmembrane domain[28] or TRAIL receptor peptides which are capable of binding to TRAIL.

The TRAIL receptor molecule may be selected from TRAIL receptor-1, (TRAIL-R1, DR4), TRAIL receptor-2 (TRAIL-R2, Apo2, DR5, KILLER, TRICK2a, TRICK2b), TRAIL receptor-3 (DCR1), TRAIL receptor-4 (DCR2, TRUND) and OPG (osteoprotegerin). Preferred are the TRAIL receptor-1 and the TRAIL receptor-2, more preferred is the TRAIL receptor-2. TRAIL receptors are described in WO 98/32856, WO 98/35986, WO 98/41629, WO 99/10484, WO 00/66156 and reference 28, which are incorporated herein by reference. It should be noted that the invention also encompasses the use of combinations of several TRAIL receptors as described above.

Especially preferred is a TRAIL inhibitor which comprises an extracellular domain of a TRAIL receptor molecule. e.g. amino acids 1-208, 1-207, 1-206, 1-188 or 1-187 of the TRAIL R 2 sequence according to reference 28, wherein optionally the homologous signal peptide sequence has been replaced by a heterologous signal peptide sequence, e.g. the Igκ or the Igλ signal peptide sequence. The extracellular domain is optionally fused to a heterologous polypeptide domain, particularly a Fc immunoglobulin molecule including the CH2 and the CH3 domain and optionally the hinge region e.g. from the human IgG1 molecule or a modified Fc immunoglobulin domain such as described in US 5,925,734 having modified, e.g. deleted effector functions such as ADCC and/or CDC.

Especially preferred are fusion polypeptides comprising an optionally truncated extracellular domain of a TRAIL receptor molecule, e.g. TRAIL-R2 and a Fc immunoglobulin molecule comprising an amino acid sequence overlap of at least one amino acid between the TRAIL receptor domain and the Fc domain, i.e. there is at least one amino acid which is common to both domains. Particularly at least one carboxy terminal amino acid of the TRAIL receptor domain overlaps with at least one amino terminal amino acid of the Fc domain, more particularly the extracellular TRAIL R domain ends with S which is the first amino acid of the Fc domain.

The invention also encompasses a nucleic acid encoding the fusion polypeptide which may be operatively linked to a suitable expression control sequence.

In a further embodiment of the present invention, the inhibitor is a TRAIL receptor inhibitor which may be selected from
(a) an inhibitory anti-TRAIL receptor-antibody or a fragment thereof, and
(b) an inhibitory TRAIL fragment.

Examples of suitable inhibitory anti-TRAIL receptor antibodies, e.g. antibodies against TRAIL receptor-1, TRAIL receptor-2, TRAIL receptor-3, TRAIL receptor-4 or OPG, and inhibitory TRAIL ligand fragments are described in WO 98/35986, WO 99/10484, WO 00/73349, reference 31 and reference 32, which are incorporated herein by reference. The antibodies may be monoclonal, chimeric, humanized or human antibodies or proteolytic or recombinant antibody fragments. Especially preferred are anti-TRAIL R1 and anti-TRAIL R2 antibodies.

In a still further embodiment the inhibitor is capable of inhibiting the interaction of the Death domain of TRAIL receptor-1 or TRAIL receptor-2 with the Death domain of FADD. Examples of suitable inhibitors are antibodies or fragments which are specifically directed against the Death domain of TRAIL-R1, TRAIL-R2 or FADD. Furthermore, the inhibitor may be capable of inhibiting the interaction between the Death Effector domain of FADD and caspase-8 and/or caspase-10, thereby inhibiting death receptor, e.g. TRAIL receptor induced processes, e.g. apoptotic processes. In this context, it is referred to reference 33 and references cited therein.

In a still further embodiment of the present invention the inhibitor is a nucleic acid effector molecule. The nucleic acid effector molecule may be selected from anti-sense molecules, RNAi molecules and ribozymes which are capable of inhibiting the expression of at least one TRAIL receptor gene and/or the TRAIL ligand gene.

In a still further embodiment the inhibitor may be directed against intracellular TRAIL receptor signal transduction e.g. specific inhibitors of TRAIL receptor signal transduction or general inhibitors of apoptotic signal transduction. Examples of such intracellular inhibitors are selected from apoptosis inhibitors, particularly intracellular apoptosis inhibitors, e.g. caspase inhibitors such as caspase-3, caspase-8 or caspase-10 inhibitors, Bid inhibitors, Bax inhibitors or any combination thereof. Examples of

suitable inhibitors are caspase inhibitors in general, cf. WO 02/094263, WO 01/10383, WO 01/42216, WO 01/90070, WO 01/94351, WO 01/21600, WO 00/61542, WO 99/47545, dipeptide inhibitors (WO 99/47154), carbamate inhibitors (WO 01/72707), substituted aspartic acid acetals (WO 01/81330), heterocyclyldicarbamides (WO 02/085899), quinoline-(di-, tri-, tetrapeptide) derivatives (US 200126467), substituted 2-aminobenzamide caspase inhibitors (WO 00/55114), substituted α-hydroxy acid caspase inhibitors (WO 01/16093) inhibition by nitrosylation (WO 98/43621); CASP-1: (WO 02/000853; CASP-3: protein-inhibitors (WO 02/066050), antisense molecules (WO 01/53310), nicotinyl-aspartyl-ketones (WO 01/27085), γ-ketoacid dipeptide derivatives (WO 02/48179, WO 00/32620, WO 00/55127), CASP-8: antisense molecules (WO 01/53541), interacting proteins (WO 00/39160) CASP-9: antisense modulators (WO 02/22641); CASP2: antisense molecules (WO 02/24720); CASP-6: antisense molecules (WO 02/29066); CASP-7: antisense molecules (WO 02/22640); CASP-12 inhibitors: WO 00/59924, which are herein incorporated by reference. Further examples are mitochondrial inhibitors such as Bcl-2-modulating factor (WO 02/097094); Bcl-2 (WO 94/27426) mutant peptides derived from Bad (WO 02/20568), Bad (WO 96/13614), BH3-interacting domain death agonist (WO 98/09980), Bax inhibitor proteins (WO 98/40397), BLK genes and gene products (WO 99/50414) which are herein incorporated by reference. Further suitable intracellular modulators of apoptosis are modulators of CASP9/Apaf-1 association (WO 02/064128), antisense modulators of Apaf-1 expression (WO 02/32921), peptides for inhibition of apoptosis (WO 99/43701), anti-apoptotic compositions comprising the R1 subunit of Herpes Simplex virus (WO 00/07618), MEKK1 and fragments thereof (WO 99/41385), modulators of Survivin (WO 01/64741), modulators of inhibitors of apoptosis (WO 97/06182, WO 00/77201, WO 01/59108, WO 02/053586) and HIAP2 (WO 00/08144) which are herein incorporated by reference. Further, any combination of the above inhibitors may be used.

The inhibitor or a combination of the above inhibitors is administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific condition by suitable means. For example, the inhibitor may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficacy and toxicity may be determined according to standard protocols. The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly or intravenously, or locally, e.g. intranasally. Preferred is intravenous and/or intranasal administration.

An especially preferred combination of inhibitors is an extracellular TRAIL/TRAIL receptor inhibitor, e.g. an anti-TRAIL antibody or a soluble extracellular TRAIL receptor domain optionally fused to a heterologous polypeptide, and an intracellular TRAIL receptor signal transduction inhibitor.

Optionally, a further active ingredient is present, which may be selected from antiviral agents such as amantadine and derivatives thereof which are directed against viral transmembrane proteins, e.g. rimantadine, inhibitors of viral neuraminidases, particularly neuraminidase from influenza virus, e.g. relanza, inhibitors of the Raf-MeK-Erk signal transduction pathway, e.g. 20126 or further inhibitors as described in PCT/DE 01/01292, inhibitors of the MIKK/SEK/YMK signal transduction pathway or components of further signal transduction pathways as described in DE 10138912 and synthetic nucleoside analoga such as 3-deazaadenozine and ribavirine.

The dose of the inhibitor administered will of course, be dependent on the subject to be treated, on the subject's weight, the type and severity of the injury, the manner of administration and the judgement of the prescribing physician. For the administration of anti-TRAIL-R or L-antibodies or soluble

TRAIL-R proteins, e.g. TRAIL-R fusion proteins, a daily dose of 0,001 to 100 mg/kg is suitable.

Still a further aspect of the present invention is a method of identifying and/or characterizing inhibitors of viral infections, particularly infections by RNA viruses such as influenza or Borna disease virus, comprising determining if a compound is capable of inhibiting the TRAIL ligand/TRAIL receptor system. The inhibition of the TRAIL ligand /TRAIL receptor system preferably comprises an inhibition of TRAIL ligand/TRAIL receptor mediated apoptosis or a TRAIL ligand/TRAIL receptor mediated cell activation. Further preferred is an inhibition of the interaction between TRAIL-R1 and/or TRAIL-R2 with the FADD Death domain or an inhibition of the interaction of the FADD Death Effector domain with caspase-8 and/or caspase-10.

The method as described above may be a molecular-based assay, wherein the effect of a compound to be tested on the interaction between a TRAIL ligand and a TRAIL receptor is analyzed in a test system comprising substantially purified and isolated components, e.g. recombinant molecules. Alternatively, the method may be carried out as a cellular-based assay, wherein a suitable test cell, e.g. a test cell expressing or overexpressing a TRAIL receptor is used. Suitable molecular- and cellular-based assay systems. e.g. high throughput assay systems are known in the art.

Further, the present invention shall be explained in more detail in the following Figures and Examples.

Figure Legends

Figure 1: NF-κB signalling is important for efficient influenza virus production

a) Immunoblot analysis of IκBα degradation. A549 cell lines stably expressing vector, IKK2 EE, IKK2 KD or IκBαmut were stimulated with TNF-α (20ng/ml) and harvested after indicated times. Note that overexpressed IκBαmut shows a retardation in SDS Gel. IKK EE cell lines do not show a constitutive IκBα degradation, however, recovery of de novo IκBα expression upon stimulation is faster. This may be the basis of enhanced NF-κB signal strength consistently observed in stimulated or infected IKK2 EE cells.

b) NF-κB promoter-luciferase-reportergene-assay. Stably transduced MDCK cell lines were transfected with a luciferase-reporter gene plasmid driven by a 3xNF-κB binding site artificial promoter. 24h after transfection, cells were infected with FPV (MOI=5), and harvested 4h post infection (p.i.) to perform luciferase-assays. Each bar represents the average and standard deviation of three independent transfections.

c) IFNß promoter/enhanceosome luciferase-reportergene-assay. Stably transduced MDCK cell lines were transfected with a luciferase-reporter plasmid driven by the IFNß-promotor/enhanceosome which carries the AP-1, IRF and NF-κB binding sites responsible for inducible IFNß expression. 24h after transfection cells were infected with Influenza A virus (MOI=5), and harvested 4h p.i. to perform luciferase-assays. Each bar represents the average and standard deviation of three independent experiments.

d) Different MDCK and type-I-interferon deficient Vero cell lines were infected with FPV (MOI=1). Supernatants were collected 9h and virus yields were determined in plaque assays on MDCK cells. Data are shown as percentage of virus titers compared to the vector

control. Each bar represents the average and standard deviation of three independent experiments.

e) Experiments were performed as described in d using the different A549 cell lines infected with FPV. Data are shown as absolute plaque forming units in a logarithmic scale. Each bar represents the average and standard deviation of three independent experiments.

Figure 2: NF-κB acts proapoptotic in A549 cell lines in the context of an influenza virus infection.

a) Immunoblot for PARP-cleavage as an early apoptotic marker. A549 cell lines were mock or FPV (MOI = 1) infected and lysed 24h p.i in Triton X-100 lysis buffer. After SDS-Page and blotting, membranes were subjected to Western blot using an anti-PARP mAb detecting both the cleaved and the uncleaved form of the protein. Erk blots served as a loading control.

b) A549 cell lines were infected with FPV (MOI = 1) and incubated with different concentration of the broad band caspase-inhibitor Z-VAD-FMK or an inactive control (Z-FA-FMK). 24h p.i. supernatants were collected and virus titers were determined.

c) Experiment was performed as in b) with different Vero cell lines infected with FPV (MOI = 1).

Figure 3: The pro-apoptotic protein TRAIL is induced upon influenza A virus infection

a) A549 cell lines were either mock-treated or infected with FPV (MOI = 1). 24h p.i. RNA was isolated and subjected to a RNase protection assay.

b) Immunoblot analysis of TRAIL expression in A549 (wild-type) cells. A549 cells were infected with FPV (MOI = 10) and lysed at different times in Triton X-100 lysis buffer. Viral NP accumulation was used as control for the infection progress.

c) Different A549 cell lines were infected with FPV (MOI = 5) and treated with monensin to prevent protein secretion. 8h p.i. cells were fixed with 4% paraformaldehyde and were stained for TRAIL expression which was detected in FACS analysis. Grey line: uninfected cells, black line: virus infected cells.

Figure 4: TRAIL is an important mediator for influenza A virus propagation.

a) Soluble TRAIL-R2-Fc prevents TRAIL-mediated caspase activation. A549 cells were either left untreated or stimulated with recombinant flag-tagged human TRAIL (20ng/ml) with or without soluble TRAIL-receptor 2 (TRAIL-R2-Fc, 10μg/ml) for 16h. Lysates were subjected to an anti-PARP Western blot as a measure for apotosis induction.

b) Presence of TRAIL-R2-Fc results in decreased influenza virus production. A549 wildtype cells were infected with FPV (MOI = 1). After infection TRAIL-R2-Fc (10μg/ml) or TNF-R2-Fc (10μg/ml) as control, was added to the medium. After 9h and 24h p.i. supernatants were collected to determine virus titers.

c) TNF-R2-Fc prevents TNFα induced IκBα degradation. A549 cells were either left untreated or stimulated with recombinant TNFα (20ng/ml) in the presence or absence of TNF-R2-Fc (10μg/ml).

d) A549 cell lines were infected with FPV (MOI = 1). 6h p.i. recombinant flag-tagged human TRAIL (10ng/ml) was added to the infection medium. 9h p.i. supernatants were collected and assayed to determine viral titers. Each bar represents the average and standard deviation of three independent experiments.

Examples

1. Methods

1.1 Viruses, cell lines and viral infections

Avian influenza virus A/Bratislava/79 (H7N7; fowl plague virus (FPV)) was taken from the virus strain collection of the Institute of Virology, Giessen, and was used for infection of different cell lines. Madin-Darby canine kidney (MDCK) cells and african green monkey kidney cells (Vero) were grown in minimal-essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. A549 human lung carcinoma cells were grown in Ham´s F12 medium supplemented with 10% heat-inactivated FBS and antibiotics. A549, MDCK and Vero cell lines stably expressing transdominant mIκBα as well as constitutively active and dominant negative IKK2 were generated with a retroviral transduction approach using the pCFG5-IEGZ retroviral vector system[27] and amphotrophic Phoenix producer cell lines essentially as described in Denk et al.[16]. For infection cells were washed with PBS incubated with virus at the indicated multiplicities of infection (MOI) diluted in PBS/BA (PBS containing 0,2% BSA, 1mM $MgCl_2$, 0,9mM $CaCl_2$, 100U/ml penicillin and 0,1mg/ml streptomycin) for 1h at room temperature. The inoculum was aspirated and cells were incubated with either MEM or Ham´s F12 containing 0,2% BSA and antibiotics. 9h or 24h p.i. supernatants were collected to assess the number of infectious particles (plaque titers) in the samples. Briefly, MDCK-cells grown 90% confluent in 6-well dishes were washed with PBS and infected with serial dilutions of the supernatants in PBS/BA for 1h at 37°C. The inoculum was aspirated and cells were incubated with 2ml MEM/BA (medium containing 0.2% BSA and antibiotics) supplemented with 0.6% Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech) and 1.5% $NaHCO_3$ at 37°C, 5% CO_2 for 2-3 days. Virus plaques were visualised by staining with neutral-red.

1.2 Inhibitors, antibodies and reagents

Caspase-inhibitor Z-VAD-FMK or inhibitor control Z-FA-FMK (Alexis Biochemicals) were supplied ready-to-use 2mM in DMSO. DMSO was used as solvent control at a final concentration of 2%, representing highest inhibitor concentration. The mouse anti-PARP monoclonal antibody was purchased from Transduction Laboratories. For TRAIL Western blots a monoclonal antibody (mAb) against human TRAIL was purchased from Santa Cruz Biotechnology (sc-8440). MAbs against TRAIL and TRAIL-R1 and R2 for FACS, as well as recombinant Flag-tagged human TRAIL are available from Alexis Biochemicals, Grünberg, Germany. Soluble TRAIL-receptor 2 (TRAIL-R2-Fc) and soluble TNF-α receptor (sTNF-R2-Fc) were produced as fusion proteins with human IgG-Fc essentially as described in reference 28. The soluble TRAIL-R2-Fc fusion domain contains amino acids 1-208 from the extracellular domain of TRAIL-R2 and the Fc domain of human IgG1 contains the hinge, CH2 and CH3 domains.

1.3 Plasmids, transfections and Western blots

The IFNß-promotor-luciferase plasmid was kindly provided by J.Hiscott, Montreal, Canada. The 3xNF-κB reporter plasmid was described previously 13. MDCK cells were transfected with Lipofectamine 2000 (LifeTechnologies) according to a protocol by Basler et al.[29]. Luciferase-reporter gene assays were carried out as described earlier[25,30]. For Western blots cells were lysed in Triton lysis buffer (20mM Tris/HCl, pH7.4, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 50mM sodium glycerolphosphate, 20mM sodium pyrophosphate, 5μg/ml leupeptin, 1mM sodium vanadate, 5mM benzamindine) on ice for 10-20 min. Cell lysates were then centrifuged and protein contents in supernatants were estimated employing a protein dye reagent (BIO-RAD laboratories). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted on nitrocellulose membranes.

1.4 Flow cytometry analysis

TRAIL was detected by an intracellular staining procedure. A549 cell lines were infected with FPV at an MOI of 5 for 8h in the presence of 2μM monensin to avoid protein secretion. Cells were fixed with 4% paraformaldehyde at 4°C for 20 min and subsequently washed twice in permeabilization buffer (0.1% saponin/1% fetal calf serum/PBS). After incubation with a mouse monoclonal antibody against TRAIL or isotype control antibodies (Becton Dickinson) cells were stained with biotin-Sp-conjugated goat anti-mouse IgG (Dianova) and streptavidine-Cy-chrome (Becton Dickinson). Fluorescence was determined in the FL3-channel using a FACScalibur cytometer (Becton Dickinson). All FACS analyses were repeated at least twice and revealed essentially similar results.

1.5 RNase Protection Assay

A549 cell lines were mock infected or infected with FPV at a MOI of 1. 24h p.i. cells were lysed and RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturers instructions. Purified RNA was subjected to the RiboQuant Multi-Probe RNase Protection Assay hAPO-3 (BD-Pharmingen) according to the manufacturers instructions.

2. Results

To analyse the role IKK and NF-κB activation during an influenza virus infection we established host cell lines which stably express dominant negative IKK2 (IKK KD), a constitutively active IKK2 (IKK EE), or a non-degradable mutant of IκBα (IκBαmut)[16] which prevents NF-κB activation. Figure 1 shows that expression of IKK KD or IκBαmut resulted in an efficient block of NF-κB activation. TNF-α-induced IκBα degradation (Fig.1a) or influenza virus-induced transcription from a NF-κB dependent promoter element (Fig. 1b) are impaired in the presence of the mutants. Although high expression of IKK EE is not well tolerated in the cell lines and the transgene is only barely detectable after a few passages (data not shown), enhanced NF-κB-dependent transcriptional activity in the IKK EE cell lines

was still detected upon virus infection. When the artifical NF-κB reporter gene construct was replaced by a reporter gene plasmid harbouring the IFNß promoter/enhanceosome the different mutants showed essentially the same effects on transcriptional activation (Fig. 1c), although less pronounced due to involvement of other virus-induced transcription factors for IFNß expression. This is consistent with earlier reports suggesting that NF-κB regulates IFNß expression as an antiviral function[2].

However, when we studied efficiency of influenza virus production in these different cell lines we found different results. Virus propagation was impaired upon NF-κB inhibition and enhanced in cells expressing the active form of IKK EE (Fig. 1d,e). The effects were similar in Madine Darby canine kidney (MDCK) epithelial cells and in Vero cells (Fig 1d). The latter cell line does not express type 1 interferons [17] excluding a prominent involvement of these cytokines in the observed effects. Inhibition or enhancement of virus propagation correlated with the efficacy of transgene action (data not shown) and was strongest in A549 lung epithelial cells, where the differences in virus titers between the different cell lines were up to 10-fold (Fig. 1e). These results differ from studies obtained upon infections of the respective NF-κB-defective CRL cell lines with Borna disease virus (BDV), another negative-strand RNA virus. Here, inhibition of NF-κB had no pronounced effect on replication, however, activation of the pathway by expression of IKK EE resulted in a strongly impaired virus replication due to a massive induction of type I interferons (O. P. and S. L., unpublished data). This suggests a crucial function of NF-κB specifically for influenza virus replication, which appears to occur independently of a type I interferon response.

NF-κB is a transcription factor, thus, the underlying mechanism most likely involves regulation of a proviral acting factor. Since NF-κB is a regulator of both pro- and antiapoptotic genes 6 we examined viral induction of apoptosis in the different NF-κB cell lines. To monitor an early apoptotic

event we chose proteolytic cleavage of a major caspase substrate, poly (ADP-ribose) polymerase (PARP). Influenza virus infection leads to a significant cleavage of PARP in vector cell lines which was further enhanced in IKK EE expressing cells (Fig. 2a). In contrast, virus-induced PARP cleavage was impaired in infected cells expressing transdominant negative mutants of IKK or IκBα (Fig. 2a). This surprising finding indicates that NF-κB acts in a proapoptotic rather than an antiapoptotic fashion in the context of an influenza virus infection. In fact apoptosis induction and caspase activation appear to be required for efficient virus propagation. Virus yields were strongly impaired in the presence of the pan-caspase inhibitor Z-VAD-FMK, but not by the inactive analogue Z-FA-FMK, both in A549 and in Vero cells (Fig. 2b,c).

Taken together, the data suggested that during influenza virus infection NF-κB upregualtes a proapoptotic factor which results in enhanced virus production. Therefore, we examined virus-induced gene expression of a variety of apoptosis regulators, such as caspase 8, Fas (APO-1, CD95), death receptor 3 (DR3), TRAIL or TNF-R1 in vector, IKK EE or IKK KD expressing cell lines using RNAse protection assays (Fig. 3a). Intriguingly, the only one of these genes that was strongly upregulated upon virus infection in vector-transfected or IKK EE expressing cell lines but completely absent in cell lines expressing IKK KD was that for the TNF-related apoptosis-inducing ligand (TRAIL). TRAIL was expressed in increasing amounts over time during an influenza virus infection (Fig. 3b) and inhibition of NF-κB signalling by IKK KD or mIκBα resulted in a complete block of virus induced TRAIL synthesis (Fig. 3c).

To test whether TRAIL might be involved in NF-κB dependent enhanced influenza virus replication we determined virus titers in the absence or presence of TRAIL-R2-Fc, an efficient inhibitor of TRAIL[28]. This reagent not only efficiently blocked TRAIL-induced PARP cleavage (Fig. 4a) but also resulted in an approximately 80% decrease of virus production after 24h

(Fig. 4b). This indicates that TRAIL is indeed a proviral factor. In contrast, the presence of soluble TNF-R2-Fc, a receptor for another proapoptotic and virus-induced cytokine, did not lead to a significant reduction of virus titers (Fig. 4b), although the same concentration of the reagent efficiently blocked TNFα-induced IκBα degradation (Fig. 4c). Consistent with the finding that TRAIL supports virus propagation, stimulation of infected cells with low concentrations of recombinant human TRAIL enhanced virus production in A549 cells and rescued the phenotype of NF-κB deficiency in the different cell lines (Fig. 4d).

Thus, the apoptosis inducer TRAIL is a proviral factor that is induced in influenza virus infected cells in a NF-κB-dependent manner.

3. Discussion

Although the role of NF-κB in influenza virus infected cells has never been studied in detail, it was a common thought that both NF-κB activation and apoptosis induction are antiviral responses to influenza virus infections. According to the data presented here we face a scenario in which NF-κB rather acts proviral in the context of an influenza virus infection at least by regulating expression of the proapoptotic factor TRAIL.

Little is known about the transcriptional regulation of TRAIL so far. However, recent studies demonstrated that expression of TRAIL can be indeed induced in a NF-κB dependent manner in Jurkat T-cells mediated through a c-Rel binding site in the proximal TRAIL promoter[18]. We now show that this is also true for a viral inducer in epithelial cells. Our data are supported by findings from a recent transcriptional profiling approach identifying TRAIL among 84 out of 13.000 genes which were deregulated in response to infection with a human influenza virus[19]. Furthermore, TRAIL and/or TRAIL-R1/2 have been shown to be upregulated during infections with several other viruses[20-23] and it has been proposed that this is an antiviral response to selectively kill infected cells or cells of the immune

system. However, our data clearly indicate that in the case of influenza virus infection NF-κB dependent TRAIL induction and subsequent TRAIL mediated apoptosis are proviral events. It therefore seems that influenza virus has acquired the capability to take advantage of the host cells protection machinery and thereby supporting viral replication. It is easier for a viral invader to take advantage of existing cellular activities rather than actively inducing such processes in the host-cell. In line with this assumption the virus needs mechanisms to keep a balance between limitation of the antiviral response and maintenance of sufficient signalling strength to support virus growth. Such a balance control may be provided by the viral NS1 protein which keeps activities of certain transcription factors in a tolerated limit[3,24,25] thereby preventing an overflow of an antiviral response but still allowing some proviral proteins to be produced. In support of such a model we observed that recombinant TRAIL only enhanced virus propagation if cells were stimulated late in infection in concentrations up to 10-20 ng/ml (Fig. 4d). Earlier stimulation or higher concentrations of TRAIL resulted in a loss of the supportive effects (data not shown). This late requirement of TRAIL correlates well with the expression kinetics of the protein during the virus replication cycle (Fig. 3c).

Furthermore, the recent finding that influenza viruses express a proapoptotic protein, PB1-F2 late in the virus life cycle is consistent with a requirement of apoptosis induction for efficient virus growth[26]. We have identified NF-κB and TRAIL as crucial cellular factors for influenza virus replication. Our findings suggest that the pharmacological inhibition of NF-κB and/or more specifically of TRAIL may be of therapeutic value in the defense against a virus which has been and still is one of the biggest threats to human health worldwide.

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Claims

1. Use of inhibitors of the TRAIL ligand/TRAIL receptor system for the manufacture of a medicament for the prevention or treatment of viral infections.

2. The use of claim 1 for the prevention or treatment of influenza or Borna disease virus infections.

3. The use of claim 1 for the prevention or treatment of influenza virus infections.

4. The use of any one of claims 1-3 for the prevention or treatment of viral infections in humans.

5. The use of any one of claims 1-3 for the prevention or treatment of viral infections in domestic or wild animals.

6. The use of any one of claims 1-5 wherein the inhibitor is a TRAIL ligand inhibitor.

7. The use of claim 6 wherein the TRAIL ligand inhibitor is selected from
   (a) an inhibitory anti-TRAIL-ligand-antibody or a fragment thereof, and
   (b) a soluble TRAIL-receptor molecule or a TRAIL ligand-binding portion thereof.

8. The use of claim 7 wherein the TRAIL-receptor molecule is selected from TRAIL receptor-1, TRAIL receptor-2, TRAIL receptor-3, TRAIL receptor-4 and OPG (osteoprotegerin).

9. The use of claims 8 or 9 wherein the TRAIL ligand inhibitor is an extracellular domain of a TRAIL receptor molecule optionally fused to a heterologous polypeptide domain.

10. The use of claim 9 wherein the TRAIL ligand inhibitor is an extracellular domain of a TRAIL receptor molecule fused to a Fc immunoglobulin molecule.

11. The use of any one of claims 1-5 wherein the inhibitor is a TRAIL receptor inhibitor.

12. The use of claim 11 wherein the TRAIL receptor inhibitor is selected from

(a) an inhibitory anti-TRAIL receptor-antibody or a fragment thereof; and

(b) an inhibitory TRAIL ligand fragment.

13. The use of any one of claims 1-5 wherein the inhibitor is a nucleic acid effector molecule.

14. The use of claim 13 wherein the nucleic acid effector molecule is selected from anti-sense molecules, RNAi molecules and ribozymes.

15. The use of any one of claims 1-5 wherein the inhibitor is an inhibitor of intracellular TRAIL receptor signal transduction.

16. The use of any one of claims 1-5 wherein the inhibitor is an inhibitor of the interaction of the Death domain of TRAIL receptor-1 or TRAIL receptor-2 with the Death domain of FADD or an inhibitor of the interaction of the Death Effector domain of FADD with caspase-8 and/or caspase-10.

17. The use of any one of claims 1-16 wherein the medicament comprises at least one inhibitor as the active ingredient together with pharmaceutically acceptable carriers, diluents and/or adjuvants.

18. The use of any one of claims 1-17 wherein the medicament comprises a further active ingredient.

19. A method of identifying and/or characterizing inhibitors of viral infections comprising determining if a compound is capable of inhibiting the TRAIL/TRAIL receptor system.

20. The method of claim 19 wherein the inhibition comprises an inhibition of TRAIL/TRAIL receptor mediated apoptosis.

21. The method of claim 19 wherein the inhibition comprises an inhibition of TRAIL/TRAIL receptor mediated cell activation.

22. A fusion protein comprising the extracellular domain of a TRAIL receptor and a Fc immunoglobulin domain wherein there is at least one amino acid overlap between the TRAIL receptor domain and the Fc domain.

23. A nucleic acid encoding the fusion protein of claim 22.

24. The nucleic acid which is operatively linked to an expression control sequence.

25. A pharmaceutical composition comprising the fusion protein of claim 22 as an active ingredient.

Abstract

The present invention relates to the use of inhibitors of the TRAIL ligand/TRAIL receptor system for the manufacture of a medicament for the prevention or treatment of viral diseases, particularly for the prevention or treatment of influenza or Borna disease virus infections.

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Figure 1

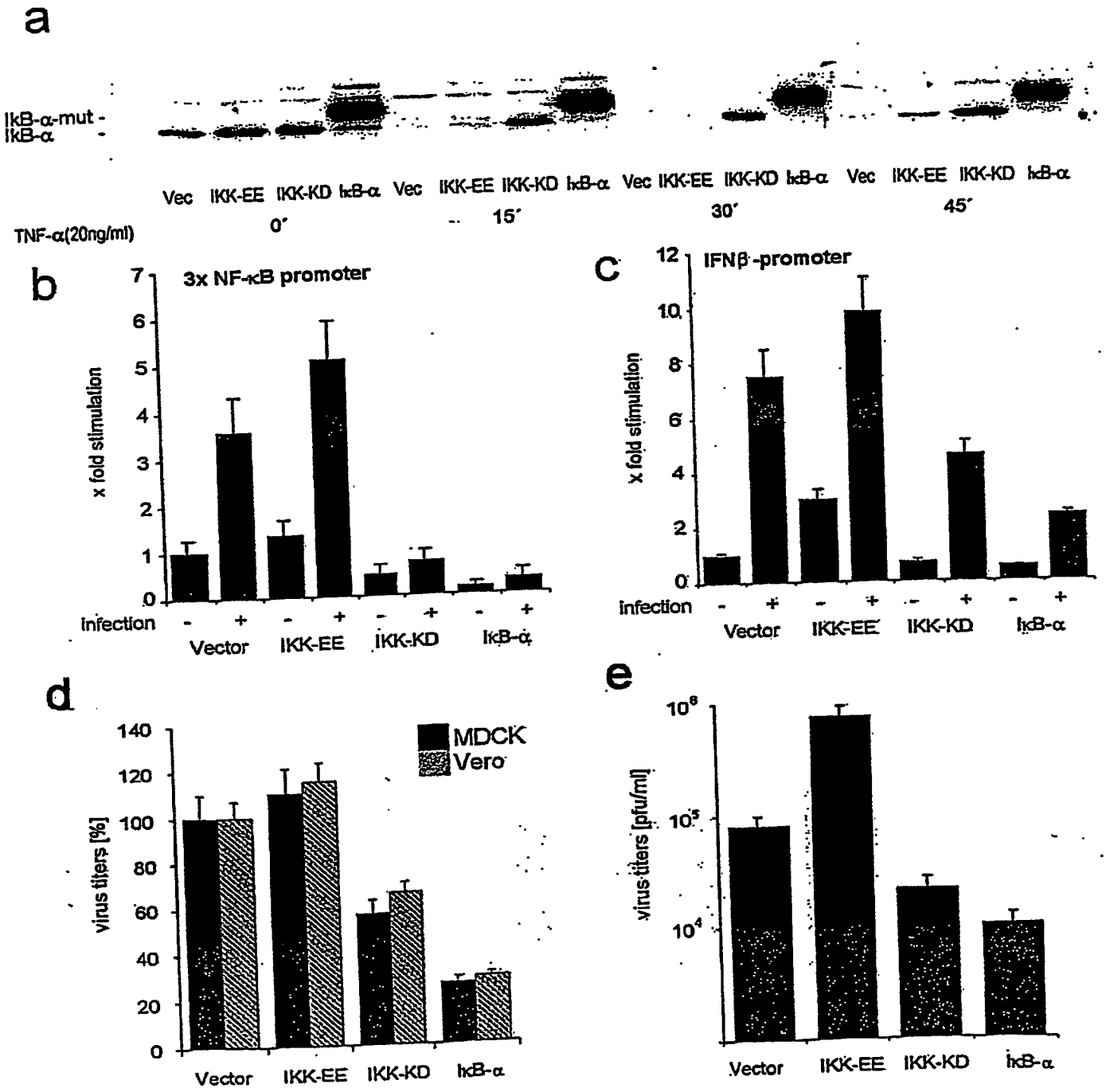


Figure 2

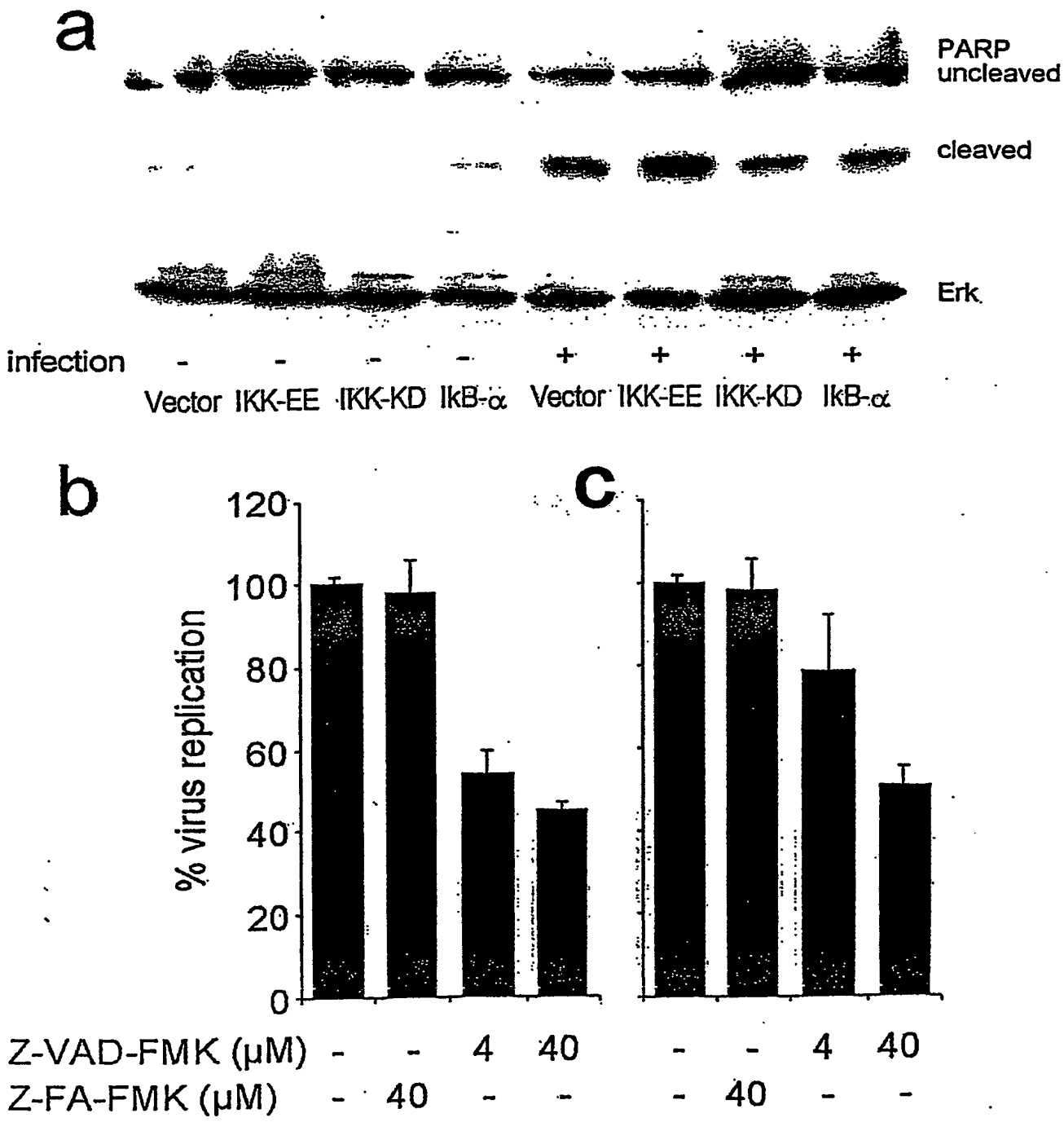
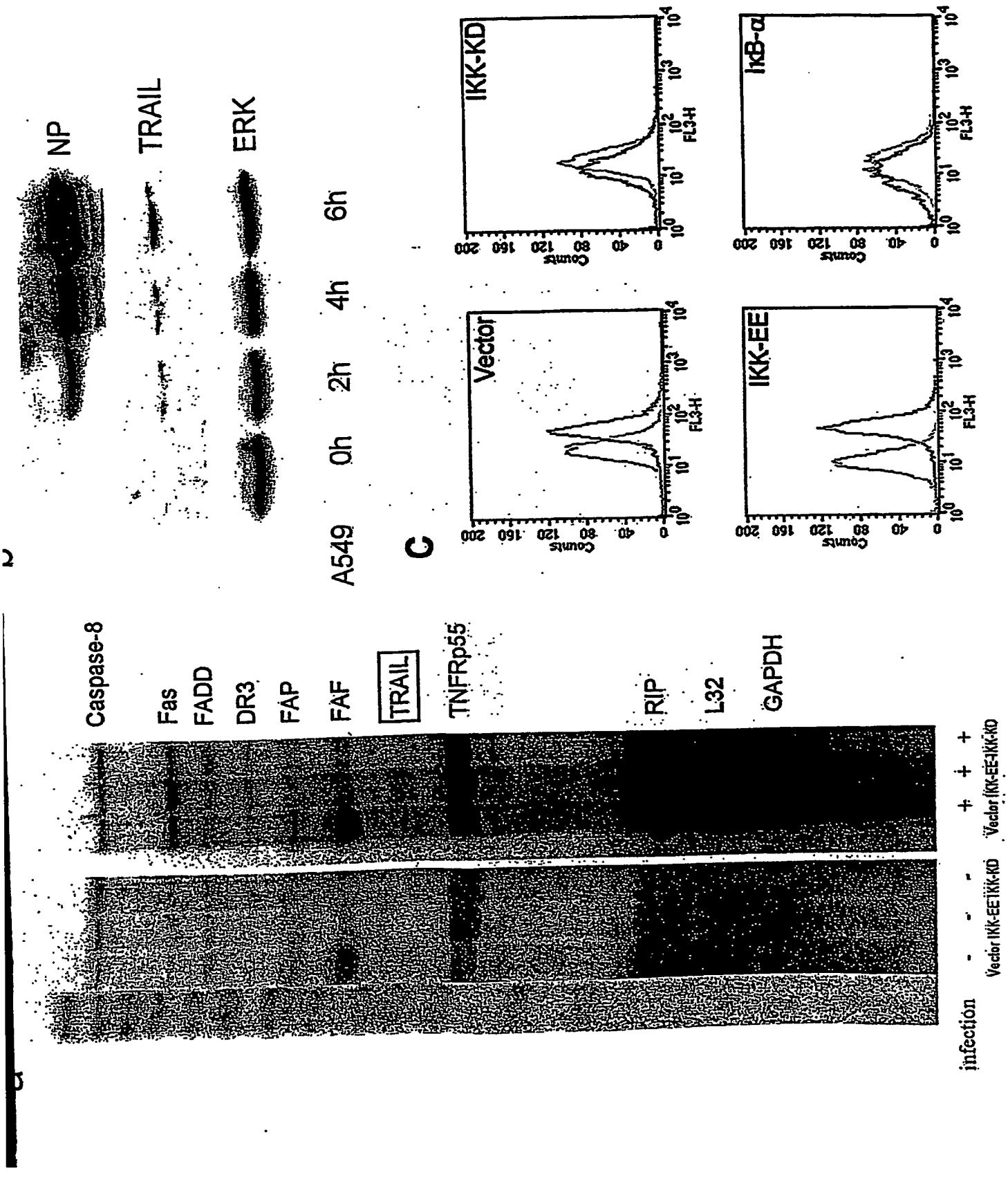

a
PARP uncleaved
cleaved
Erk
infection - - - - + + + +
Vector IKK-EE IKK-KD IkB-α Vector IKK-EE IKK-KD IkB-α
b
c
% virus replication
120
100
80
60
40
20
0
Z-VAD-FMK (μM) - - 4 40 - - 4 40
Z-FA-FMK (μM) - 40 - - - 40 - -

Figure 3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C
IKK-KD
IκB-α
Vector
IKK-EE
Counts
FL3-H
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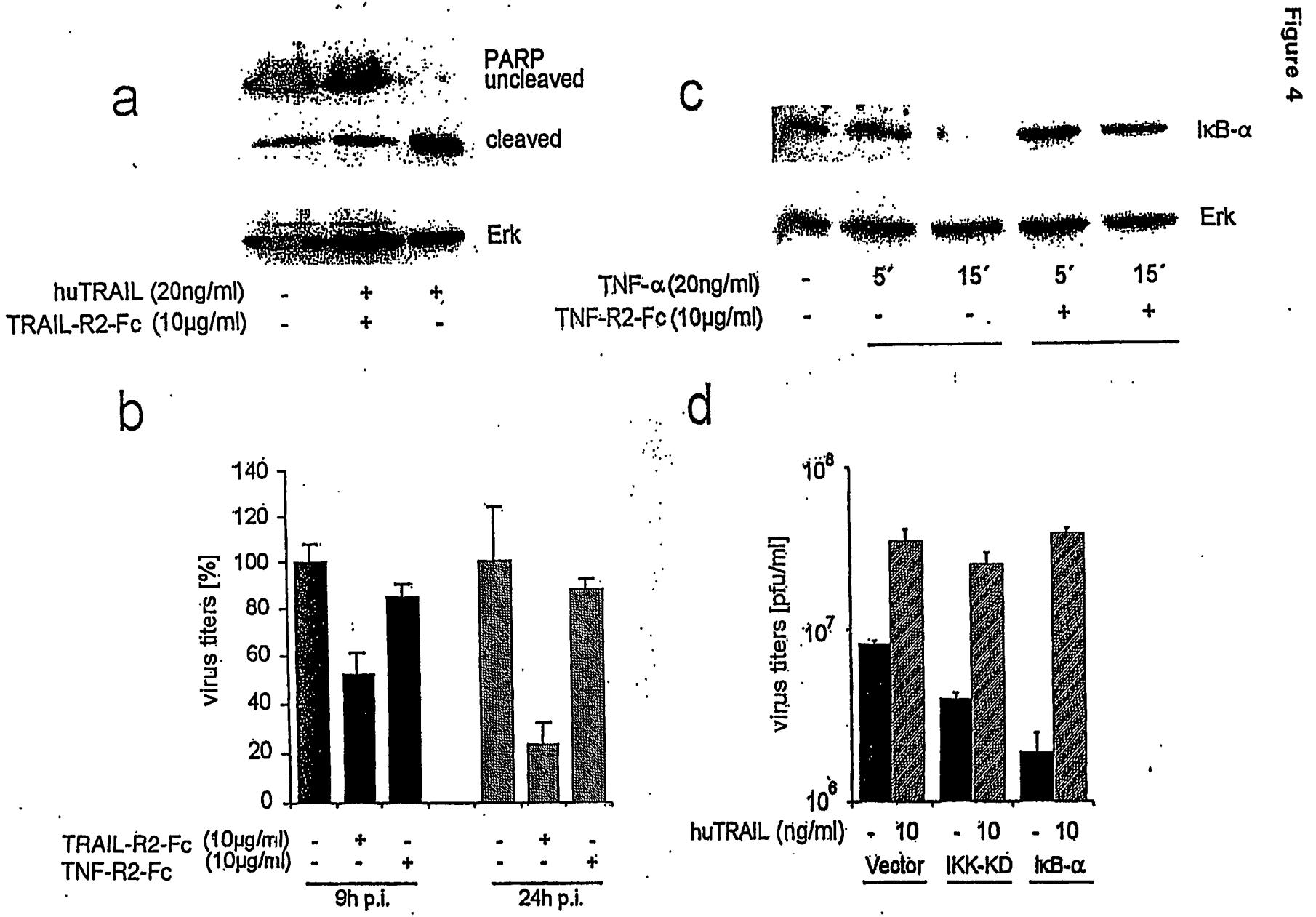
Fas
FADD
DR3
FAP
FAF
TRAIL
TNFRp55
RIP
L32
GAPDH
Infection - - - + + + +
Vector IKK-EE IKK-KD Vector IKK-EE IKK-KD

Figure 4


a
PARP
uncleaved
cleaved
Erk
huTRAIL (20ng/ml) - + +
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c
IκB-α
Erk
TNF-α (20ng/ml) - 5′ 15′ 5′ 15′
TNF-R2-Fc (10µg/ml) - - - + +
b
virus titers [%]
140
120
100
80
60
40
20
0
TRAIL-R2-Fc (10µg/ml) - + - - + -
TNF-R2-Fc (10µg/ml) - - + - - +
9h p.i.
24h p.i.
d
virus titers [pfu/ml]
10^8
10^7
10^6
huTRAIL (ng/ml) - 10 - 10 - 10
Vector
IKK-KD
IκB-α